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A network-based approach for predicting Hsp27 knock-out targets in mouse skeletal muscles

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Abstract: Thanks to genomics, we have previously identified markers of beef tenderness, and computed a bioinformatic analysis that enabled us to build an interactome in which we found Hsp27 at a crucial node. Here, we have used a network-based approach for understanding the contribution of Hsp27 to tenderness through the prediction of its interactors related to tenderness. We have revealed the direct interactors of Hsp27. The predicted partners of Hsp27 included proteins involved in different functions, e.g. members of Hsp families (Hsp20, Cryab, Hsp70a1a, and Hsp90aa1), regulators of apoptosis (Fas, Chuk, and caspase-3), translation factors (Eif4E, and Eif4G1), cytoskeletal proteins (Desmin) and antioxidants (Sod1). The abundances of 15 proteins were quantified by Western blotting in two muscles of HspB1-null mice and their controls. We observed changes in the amount of most of the Hsp27 predicted targets in mice devoid of Hsp27 mainly in the most oxidative muscle. Our study demonstrates the functional links between Hsp27 and its predicted targets. It suggests that Hsp status, apoptotic processes and protection against oxidative stress are crucial for *post-mortem* muscle metabolism, subsequent proteolysis, and therefore for beef tenderness.

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Introduction

Tenderness, flavour, juiciness, and marbling are very important attributes in the determination of beef quality even if payment on the basis of beef quality exists only in Australia at this moment. Among these attributes, there is specific attention to tenderness, which is the top priority quality attribute in beef [1]. A better control of beef tenderness is of major importance for beef producers and retailers in order to satisfy the consumers' requirement for a consistently satisfactory product [2]. For this reason, the beef industry is looking for biological markers that would identify live animals with desirable quality attributes, in order to orientate them towards the most appropriate production systems. However, tenderness is highly variable partly due to the nature of muscle, which is a complex biological structure, consisting of fibres, adipocytes and connective tissue with different properties [3,4]. Tenderness is also highly dependent on mechanisms occurring during the *post-mortem* transformation of muscle [5].

Transcriptomic and proteomic studies including ours [6,7] have attempted to identify gene affecting phenotypic differences for tenderness in cattle using high-density microarrays and two-dimensional electrophoresis [6]. They have identified some potential biological markers of beef tenderness in different production systems. These biomarkers are involved in a lot of different cellular pathways such as muscle contraction, stress reactions, glycolysis and apoptosis [8]. In order to further understand the functional relationships between these markers that may participate in controlling tenderness, we computed a bioinformatic analysis [9]. It allowed the construction of a first "tenderness network" consisting of 330 proteins based on 24 initial biomarkers of beef tenderness. In this network, heat shock proteins and especially the Hsp27 were found at crucial nodes [9]. Hsp27 is encoded by the HspB1 gene and belongs to the small heat shock family also called Hsp20 family, comprising the Hsp20, Hsp27, and αβ-crystallin. Interestingly, several studies have shown that Hsp27 expression is correlated with tenderness and could be used as a tenderness biomarker [6,10-12] . Its role in tenderness could be achieved partly through apoptosis and be correlated with its phosphorylation and oligomeric size [13].

Hence, the aim of the present study was to analyze the consequences of the targeted invalidation of the HspB1 gene on the proteins interacting with Hsp27 and linked to beef tenderness. We performed a network analysis to reveal the partner proteins of Hsp27. Then, we analyzed their abundance in the muscle of HspB1-null mice and their controls. The study enabled the identification of several pathways potentially involved in the determination of tenderness.

Materials and methods

Bio-informatics

The first part of the work was devoted to the identification of proteins that interact with Hsp27 according to information stored and shared in bioinformatic databases. This was performed using the software for systems biology Pathway Studio (Ariadne Genomics). Pathway Studio helps to interpret experimental data in the context of pathways, gene regulation networks, protein interaction maps, and to automatically update pathways with newly published facts using MedScan technology (www.elsevier.com). The Medscan reader extracts the relationship information from literature. We used the ResNet Mammalian (human, rat and mouse) database which contained the latest information extracted from the literature and

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Table 1. Suppliers and conditions for each antibody used in this study.

Target protein	Protein name	Primary antibody type	References	Dilution
Hsp27	Heat schock protein 27	Monoclonal	Santa Cruz: SC13132	1/1000
Hsp20	Heat shock protein 20	Monoclonal	Santa Cruz: SC51955	1/200
Cryab	Crystallin, alpha B	Monoclonal	Enzo: SPA-222	1/2000
Hspbap1	Heat shock protein 27-associated protein 1	Polyclonal	Santa Cruz: SC-99444	1/4000
Hsp40	Heat shock protein 40	Monoclonal	Santa Cruz: SC-56400	1/400
Hsp70a1a	Heat shock protein 70 1A	Monoclonal	R&D Systems: #242707	1/500
Hsp90aa1	Heat shock protein 90-alpha	Monoclonal	R&D Systems: #341320	1/500
Fas	Tumour necrosis factor receptor superfamily member 6, TRAF6	Polyclonal	R&D Systems: #AF 435	1/500
Chuk	Inhibitor of nuclear factor Kappa-B kinase subunit alpha	Polyclonal	Tebu-bio: E11-0441A	1/1000
Sod1	Superoxide dismutase	Polyclonal	ACRIS: APO3021PU-N	1/2000
Casp3	Caspase-3	Polyclonal	Santa Cruz: SC-7148	1/500
Cycs	Cytochrome c	Polyclonal	Tebu-bio: PAB 8027	1/10000
Eif4E	Eukaryotic translation initiation factor 4E	Monoclonal	R&D Systems: clone 299910	1/250
Eif4G1	Eukaryotic translation initiation factor 4 gamma 1	Monoclonal	Tebu-bio: H00001981-M10	1/1000
Des	Desmin	Monoclonal	DAKO: D33 M0760	1/250

from published high-throughput experiments. The approach was to build a network centred on Hsp27 interactors also called nearest neighbours. The filter options used were "protein" as entity type and "regulation" and "direct regulation" as applicable relation types. Then, the intersection between the Hsp27 neighbours and the list of 330 proteins from a previous tenderness network [9] was computed to get a list of Hsp27 interactors putatively linked to tenderness.

Animals and experimental procedure

In this study we used a constitutive knock out by gene deletion of HspB1 in mice (HspB1-null mice. This was achieved through targeted insertion (homologous recombination) as described in Kammoun *et al.* [14]. About 100 % of the HspB1 coding sequence gene was replaced by bacterial vector obtained from BMQ BAC library (Mouse Micer vector set 369N20). The commercial heterozygous ES cells (HspB1 $^{-/+}$) were microinjected into the blastocoels of mouse embryos. Embryos that received ES cells were then implanted into surrogate mothers. The resulting chimeras with a high percentage of agouti coat color were mated to wild type C57BL/6 mice to generate F1 offspring. All experiments using homozygous (HspB1 $^{+/+}$), heterozygous (HspB1 $^{-/+}$), or HspB1 homozygous null mice (HspB1 $^{-/-}$) were performed on C57BL/6 background. The F2 offspring were mated in order to amplify the three strains. Mice were housed at the experimental plant of nutrition and microbiology of the National Institute of Agronomic Research (INRA-France), in a temperature and humidity controlled room under a 12-hour light and dark cycle. They were fed *ad libitum*. Ten males were selected to constitute 2 experimental groups. Experimental procedures and animal holding respected French animal protection legislation, including licensing of experimenters. They were controlled and approved by the French Veterinary Services (agreement number CE 84-12).

Muscle samples

The HspB1-null mice were sacrificed at 12 weeks postnatal. Two muscles with different composition in fibre types were collected, namely the m. *Soleus* (slow oxidative) and the m. *Tibialis Anterior* (fast glycolytic) [15]. Muscle samples were taken immediately after sacrifice, frozen in liquid nitrogen and kept at -80 °C until protein extraction. Total protein extractions were performed according to Bouley et al. [16] in a denaturation/extraction buffer (8.3 M urea, 2 M thiourea, 1% DTT, 2% CHAPS) and stored at -20°C until use. The protein concentration was determined by spectrophotometry with the Bradford assay [17].

Immunological protein quantification

The conditions for use and specificity of primary antibodies against candidate proteins were assessed by Western blotting in order to check the specificity of all the antibodies. An antibody was considered specific when its target bands were detected at the expected molecular weight. Fourteen primary antibodies were tested for their specificity and their optimal dilution ratios were determined. Conditions used and suppliers for all primary antibodies are reported in Table 1. Secondary fluorescent-conjugated IRDye 800CW antibodies were supplied by LI-COR Biosciences (Lincoln, NE, USA) and used at 1/20000.

The abundance of candidate proteins was measured by Western blotting in the m. *Soleus* and the m. *Tibialis Anterior* of HspB1-null mice *vs* their control littermates. Fifteen µg of proteins were separated by gel electrophoresis using SDS-PAGE for 2 hr according to the Laemmli method [18]. After migration, the proteins were transferred onto PVDF transfer membrane Millipore (Bedford, MA01730, USA). Membranes were then blocked with 5% non-fat milk in TBS1 x buffer containing (blocking solution) and incubated under gentle agitation all night at room temperature in the presence of the primary antibodies. Then the membranes were incubated at 37°C for 30 minutes with the secondary fluorochrome-conjugated LICOR-antibody. Infrared fluorescence detection was then used for protein quantification. Membranes were scanned by the scanner Odyssey (LI-COR Biosciences) at 800 nm. Band volumes were quantified in the images using ImageQuant TL v 7.0.1.0 software (Amersham). Protein abundance for each sample is given in arbitrary units.